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Regulation of human genome expression and RNA splicing by human papillomavirus 16 E2 protein

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ABSTRACT

Human papillomavirus 16 (HPV16) is causative in human cancer. The E2 protein regulates transcription from and replication of the viral genome; the role of E2 in regulating the host genome has been less well studied. We have expressed HPV16 E2 (E2) stably in U2OS cells; these cells tolerate E2 expression well and gene expression analysis identified 74 genes showing differential expression specific to E2. Analysis of published gene expression data sets during cervical cancer progression identified 20 of the genes as being altered in a similar direction as the E2 specific genes. In addition, E2 altered the splicing of many genes implicated in cancer and cell motility. The E2 expressing cells showed no alteration in cell growth but were altered in cell motility, consistent with the E2 induced altered splicing predicted to affect this cellular function. The results present a model system for investigating E2 regulation of the host genome.

Introduction

Human papillomavirus 16 is a causative agent in around 50% of all cervical carcinomas and 90% of HPV positive head and neck cancers (zur Hausen, 2009). The genome of HPV16 is circular double stranded DNA of approximately 8 kbp. The E2 protein of all papillomaviruses binds as a homo-dimer to 12 bp palindromic sequences in the long control region of the viral DNA via a carboxyl terminal homo-dimerization and DNA binding domain (Desaintes and Demeret, 1996). The amino terminal domain of E2 can regulate transcription from adjacent promoters and in the case of HPV16 can either increase or decrease transcription depending upon the level of E2 protein and the cell type used for study (Bouvard et al., 1994). In addition E2 binds to 3 target sites surrounding the viral origin of DNA replication and via a protein–protein interaction recruits the viral helicase E1 (Mohr et al., 1990; Sakai et al., 1996; Yasugi et al., 1997). E1 then forms a di-hexamer that interacts with cellular DNA polymerases and initiates viral genome replication (Masterson et al., 1998). One further role for E2 in the viral life cycle is to act as the mitotic receptor for the viral genome; E2 interacts with the host genome via its amino terminus therefore tethering the viral genome to that of the host during mitosis ensuring that the viral genomes segregate to both daughter cell nuclei following cell division. Brd4 is the mitotic receptor for some but not all E2 proteins (McPhillips et al., 2006; You et al., 2004), TopBP1 is a potential cellular candidate mediating HPV16 association with mitotic chromatin (Donaldson et al., 2007).

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The large majority of studies on E2 have focused on regulation of the viral genome. This report focuses on the regulation of the host genome by HPV16 E2. Over expression of E2 can be toxic to many cell types, particularly those transformed by papillomaviruses (Desaintes et al., 1997; Goodwin et al., 1998; Parish et al., 2006). One of the mechanisms of toxicity in these cells is mediated by E2 binding and repressing the promoter controlling the E6 and E7 oncogenes that target and functionally inactivate the tumor suppressors p53 and pRb respectively. Repression of the viral oncogenes reactivates the tumor suppressor pathways resulting in cell growth inhibition and cell death (Hwang et al., 1996; Wu et al., 2000). In some of these studies micro-array experiments have been carried out to investigate the cellular genes that are regulated by the over expression of E2 (Johung et al., 2007; Morrison et al., 2011; Naeger et al., 1999; Ramirez-Salazar et al., 2011; Thierry et al., 2004). By definition all of these studies have been carried out transiently as the cells are destined for growth inhibition or cell death, and the E2 protein is delivered by viruses or plasmid over expression that will potentially add additional toxicity to the cell. E2 is also toxic to non-HPV transformed cell lines and the precise reasons for the toxicity are not certain although interaction with p53 may play a role (Parish et al., 2006). A recent study investigated the regulation of cellular genes by HPV16 E2 in C33a cells, but again this was carried out using a viral delivery system with transient expression levels of the E2 protein although clear gene changes were observed independently of the presence of other HPV proteins (Ramirez-Salazar et al., 2011). Jang et al. (2009) investigated the interaction of E2 with chromatin and demonstrated that in C33a cells E2 co-localizes with Brd4 on host chromatin around actively transcribed genes, although there was no significant difference in the RNA levels of the genes E2 associates with; it was proposed that this localization of E2 in active chromatin facilitates the viral life cycle. Another recent study investigated the E2 binding sites present in the host genome and demonstrated that HPV11 E2 can interact with some of these sites, but again when binding adjacent to active genes then the regulation of these genes was, overall, not changed (Vosa et al., 2012). This supports the work of Jang et al. (2009) and the hypothesis that E2 may bind near active genes to ensure the virus is in an open chromatin configuration allowing viral transcription and replication. Therefore, all of this work to date has not fully investigated the regulation of host genes by any E2 protein with physiologically tolerated levels of E2. This is important since it is possible that E2 assists in programming the cell to accommodate infection and the viral life cycle by directly targeting the host genome. To investigate this we used U2OS cells as a model system.

U2OS cells were derived from an osteosarcoma and have many positives for investigating the regulation of the host genome by E2. First, these cells are p53 positive and retain a p53 response following DNA damage (Allan and Fried, 1999); E2 binds to p53 and it is possible that it could regulate the p53 response (Massimi et al., 1999). Although HPV16 E6 binds to and degrades p53 other DNA virus families use multiple mechanisms to silence the p53 response and it is possible HPV does the same (Soria et al., 2010). Second, U2OS cells tolerate physiological levels of HPV16 E2; stable clones expressing E2 can be produced (Taylor et al., 2003). We would like to stress that we are not claiming the E2 levels present in the U2OS cells are totally reflective of those in HPV lesions; these levels will be widely varied from lesion to lesion. But the levels of E2 do not kill or growth arrest the cells allowing investigation of E2 genome regulation in the absence of stress and/or apoptosis pathways. Third, U2OS cells can retain HPV16 genomes as stable episomes providing a model system for screening the E2 target genes for disruption of episomal viral genome maintenance (Grant et al., 2013; Reinson et al., 2013). Fourth, U2OS cells are extremely tractable for future studies in determining how E2 regulates host genes. ENCODE data has already been generated on histone modification patterns in U2OS cells and therefore future studies investigating the E2 interaction with the host chromatin and modification of histone modifications using ChIP-seq are technically achievable in this cell line (Grant et al., 2013).

Here we report that, in stable U2OS clones expressing HPV16 E2, the cellular genome is altered at the level of gene expression and exon splicing. These changes affect genes associated with cervical cancer progression, as well as cell motility. U2OS represents a model system for studying the effect of E2 on the host genome.

Results

Generation of stable U2OS cells expressing HPV16 E2

In order to establish U2OS cells expressing HPV16 E2 (which from now on will be designated "E2" as all results presented are for HPV16 E2) a similar protocol to that previously published was carried out (Taylor et al., 2003) (the older cell lines expressing E2 were established over 10 years ago so fresh lines were prepared). Stable colonies following transfection were isolated and expanded and screened for E2 expression. Fig. 1a shows expression of E2 in two of these lines that were chosen for further studies. From day to day the expression of E2 did not affect the growth of the U2OS cells as demonstrated using a cell growth curve with the two clones (Fig. 1b).

Identifying differentially expressed host genes induced by E2

To investigate the regulation of the host genome by E2, RNA was prepared and used for Affymetrix exon array studies. The protocol

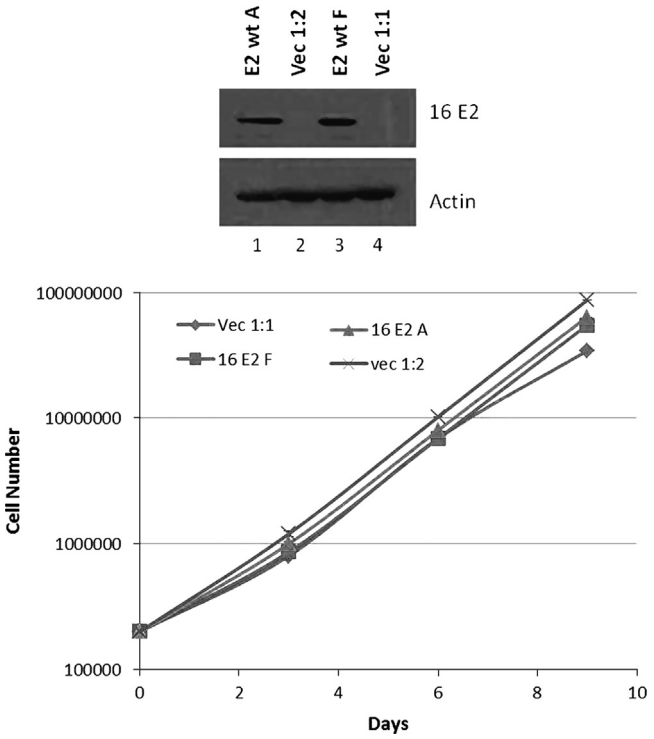

Fig. 1. Initial characterization of HPV16 E2 expressing cells. U2OS clones expressing E2 were generated and expression of E2 confirmed using western blotting (a). Clearly in lanes 1 and 3 there is E2 protein detectable; actin is used as a loading control. To determine whether the expression of E2 had an effect on cell proliferation cell growth curves were carried out (b). The two E2 expressing clones behave very similarly to the two vector control clones demonstrating that E2 does not alter the growth of cells. Error bars are included in this figure but as it is a log scale they are too small to appear; the standard errors were relatively small. The highest standard error varied 5% from the average but was ordinarily a lot lower.

for preparing the RNA, processing, screening and analyzing the data is given in "Materials and methods"; the experiment was carried out in triplicate with freshly prepared RNA each time. Following this experiment and the data analysis 74 genes were found to be differentially regulated 2 fold or greater when E2 was compared with the vector control (p-value ≤ 0.05); 33 up-regulated and 41 down-regulated. These genes are listed in Table 1. Ten of the altered genes were chosen to validate the array results in two independent clones of E2 and the results are shown in Table S1. In E2 clone F (which was used for the exon array experiments), 7 out of 10 gene changes were validated while in E2 clone A, 10 out of 10 tested validated. Overall this demonstrates that the results generated from the exon array screen were predictive of actual gene changes.

Table 1
Genes regulated in U2OS cells by HPV16 E2. The list of genes was generated as described in the "Materials and methods" and these genes represent those whose overall transcriptional level has been increased or decreased by the levels indicated. The results validated in the parental E2 clone and also an additional E2 clone; the detail of the validation is presented in Table S1.

Up-regulated by E2	Fold-change	Down-regulated by E2	Fold-change
HIST1H2BM	71.9508	GTSF1	−302.705
HIST1H3H	70.7304	NFE4	−24.794
MAGEC1	40.1869	TMPRSS15	−11.624
SLN	14.5889	SLC14A1	−5.94492
HOXB2	10.631	C1orf85	−4.48436
TNFAIP6	4.86318	CD33	−4.1363
ENG	4.85397	CLIC2	−3.9316
GNG11	4.11702	FBXL13	−3.60857
FAR2	3.70124	PTGFR	−3.59376
ZNF788	3.3983	CSTA	−3.49077
APBA2	2.99782	NLRP5	−3.33859
SERPINA3	2.74046	DNAJC18	−3.31614
C10orf72	2.5905	BMPER	−3.27652
GFPT2	2.53867	HBE1	−3.07671
SH3PXD2B	2.50361	RNF144B	−3.04874
FAP	2.47498	GDF15	−2.98979
LRRCC1	2.36516	LOC79015	−2.97446
ARHGDIB	2.35827	HSD17B8	−2.75366
SNTB1	2.25035	HIST1H3E	−2.69962
EML1	2.23335	MLH3	−2.64666
ARHGAP11A	2.23306	NLRP4	−2.60899
CCDC99	2.16172	TRIML2	−2.55713
FGD4	2.1616	CD68	−2.47599
ZNF271	2.15217	ACYP1	−2.43839
MTMR10	2.11787	PSG9	−2.41357
CALHM2	2.11199	HLA-DPA1	−2.38118
HNMT	2.09265	GNGT2	−2.37174
GOLGA8B	2.08224	FST	−2.35479
AQR	2.05191	GCNT2	−2.3148
NFIA	2.02545	GRAMD3	−2.29601
ZNF770	2.02363	PGAP2	−2.20441
ZADH2	2.01373	RPL23	−2.20367
RTKN2	2.00087	IL1RAPL1	−2.18097
		OR10A3	−2.14773
		GPR65	−2.14222
		AURKC	−2.13613
		ZNF300P1	−2.10876
		ME3	−2.06315
		ADRB2	−2.05351
		PSG4	−2.04519
		ZFP90	−2.02583

Analysis of the 74 genes differentially expressed using DAVID (GO Biological Process and KEGG Pathways) revealed no significant groups of gene changes likely due in part to the relatively small number of genes involved. The 74 genes were also subjected to analysis using Ingenuity Pathway Analysis software (www.ingenuity.com) which identified 5 networks, shown in Figure S1. Table 2 lists the associated functions with the networks; the presence of cancer, reproductive system disease, gene expression, DNA replication and repair would be predicted for a transcription and replication factor from an oncogenic human papillomavirus. Table S2 lists all of the genes in these networks and has an assigned significance score from IPA.

Over-represented DNA sequences within promoters of E2-regulated genes

We analyzed the promoters of genes altered in expression by E2 for DNA sequences with statistically significant over-representation. Analysis for 7mer sequences identified numerous sequences, one of which (GGGATTA) matched the binding sites for the OBOX1 and PITX3 transcription factor binding sites (p-value$=3\times10^{-8}$, FDR$=0.00026$). This association suggests a possible interaction between E2 and these transcription factors that could result in altered expression of these genes. None of the other sites identified were potential transcription factor binding sites.

E2 induction of exon splice variation

E2 regulates splicing of HPV gene transcripts via interaction with cell RNA splicing apparatus therefore the regulation of host RNA transcript splicing by E2 was investigated (Johansson et al., 2012). Our analysis of the exon array data showed 522 genes with differentially spliced exons induced by E2, in which the fold expression of E2 over control for one or more exons deviated significantly from the mean fold expression for all exons within each transcript. A list of these genes is shown in Table S3. Most genes had only one or a few exons differentially expressed compared to the bulk of exons for each transcript. These genes did not exhibit differential expression for the gene as a whole. Fig. 2 shows two examples, the MARK2 gene and the KIF1a gene. MARK2 is a protein kinase involved in microtubule regulation (Nishimura et al., 2012). KIF1a is a kinesin motor involved in vesicle transport in hippocampal neurons (Lo et al., 2011) that has also been implicated in head and neck cancers (Demokan et al., 2010). The exon expression analysis of the MARK2 gene showed one exon induced in the presence of E2 in comparison to the 22 other exons being expressed. For the KIF1a gene there are several of the early exons that are elevated in their expression.

We analyzed the biologically associated properties of the E2-induced alternatively spliced genes using DAVID and IPA to determine if these genes were random or were part of specific pathways and biological processes. Table 3 summarizes results of the DAVID analysis demonstrating that E2 regulates splicing of genes involved

Table 2
Associated functions with IPA networks from E2 regulated genes. The 74 genes listed in Table 1 were subjected to IPA and 5 networks of genes were identified. The networks are shown in Figure S1 and the genes associated with the networks listed in Table S2. The above table lists the predicted functions associated with the IPA generated networks.

Network	Associated network functions
1	Protein synthesis; protein degradation; hereditary disorders
2	Tissue morphology; cancer; reproductive system disease
3	Gene expression; cancer; renal and urological disease
4	Hematological system development and function; organismal development; cell morphology
5	Cellular assembly and organization; DNA replication, recombination, repair; gene expression

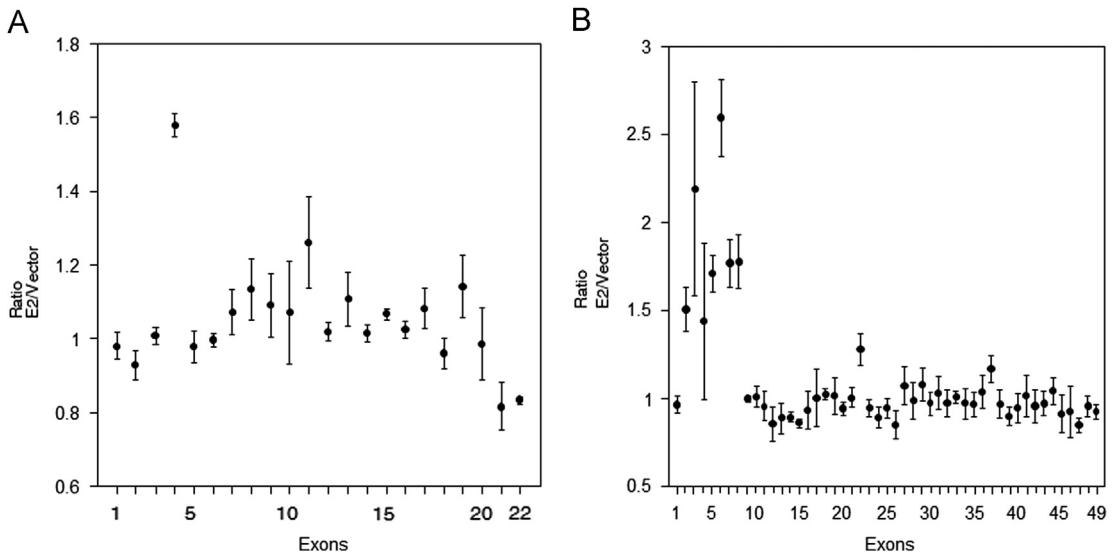

Fig. 2. An example of genes alternatively spliced in the presence of HPV16 E2. Plots of relative expression of exon probesets for (A) MARK2 and (B) KIF1A. The ratios of E2 to vector control for normalized expression values for each array exon probe are shown on the *Y*-axis for the MARK2 and KIF1A genes. The *X*-axis is labeled with the exon series probes from the exon array as defined by Affymetrix in the direction of transcription. For some array exon probes, multiple probes cover the same exon. Standard error bars based on triplicate analysis are shown. We note that while the data shown is displayed as ratios, the statistical analysis to identify genes with differentially spliced RNAs was based on Log_2-transformed ratios.

Table 3
DAVID analysis of genes differentially spliced in the presence of E2. The source of the analysis is given in the left hand column and the affected process in the right. A list of genes associated with each process is listed in Table S4.

Source	Process or pathway
KEGG pathway	Focal adhesion
GO biological process	Protein amino acid phosphorylation
GO biological process	Phosphate metabolic process
GO biological process	Phosphorus metabolic process
GO biological process	Phosphorylation
KEGG pathway	Regulation of actin cytoskeleton
KEGG pathway	Viral myocarditis
GO biological process	Actin cytoskeleton reorganization
KEGG pathway	Insulin signaling pathway
KEGG pathway	Ubiquitin mediated proteolysis
KEGG pathway	Phosphatidylinositol signaling system

adhesion/motility processes, and cytoskeletal re-organization. The genes associated with these functions are presented in Table S4. In addition, the genes with E2-induced alternatively spliced transcripts showed a statistically significant association with cancer genes from the Network of Cancer Genes (http://bio.ieo.eu/ncg/). The cancer genes are listed in Table S5.

Regulation of cell motility by E2

The exon analysis demonstrates that E2 alters splicing in genes implicated in cell movement, and there were also genes implicated in cell movement and motility altered in the fold gene expression change analysis (Table 1). SH3PXD2B regulates EGF dependent cell migration (Bogel et al., 2012) and lamellipodia formation (Lanyi et al., 2011) and cell spreading. FGD4 promotes migration of nasopharyngeal carcinoma cells infected with EBV (Liu et al., 2012) while FAP is a homodimeric integral membrane gelatinase involved in promoting invasion (Kelly et al., 2012). ARHGDIB is a RhoGDP dissociation inhibitor that can regulate metastasis, potentially by down regulating adhesion (Griner and Theodorescu, 2012). All of these genes are up-regulated in the gene set in Table 1 and potentially may modulate migration of the E2 expressing cells.

To investigate whether this altered gene expression or RNA splicing in the E2 expressing cells resulted in altered cell motility, wound healing assays were carried out. The E2 expressing cells were delayed in closing the gap between cells compared with vector control cells and Fig. 3 displays the results from a representative experiment. This experiment was carried out independently three times with essentially the same results; the E2 cells were always delayed in closing of the wound. As the E2 and Vec expressing U2OS cells have the same growth rate (Fig. 1b) it was hypothesized that the delay in wound closing by the E2 expressing cells was due to altered cell movement. To further investigate this movies were made of the E2 and Vec expressing cells over a 48 h time period, the results of this are shown in Figure S2. It is clear from the movies that the E2 cells move differently when compared with the Vec cells; this result was duplicated with two independent clones. Overall the results demonstrate that the E2 expressing cells are altered in their ability to migrate.

A significant sub-set of E2 regulated genes are altered in cervical lesions

To determine whether any of the E2 regulated genes are altered in cervical lesions, existing micro-array data was investigated. Three sets of data generated from independent studies (Ng et al., 2007; Scotto et al., 2008; Zhai et al., 2007) were used and the outcome of this analysis is shown in Table 4. Table S7 contains the same set of genes with the fold differences and *p* values listed. Of the 74 genes in Table 1, 20 were regulated in the same direction in at least one of the data sets. This represents a significant percentage of the genes that are regulated by E2 and raises the possibility that E2 can program host genomes during infection. In many cervical cancers E2 expression is lost following integration of the viral genome into that of the host (zur Hausen, 2009) but it is possible that E2 lays down an epigenetic marker on genes that retains their regulation even in the absence of E2 protein.

Discussion

There have been few studies investigating the direct role of E2 in regulating host gene expression outside of the HPV cell line

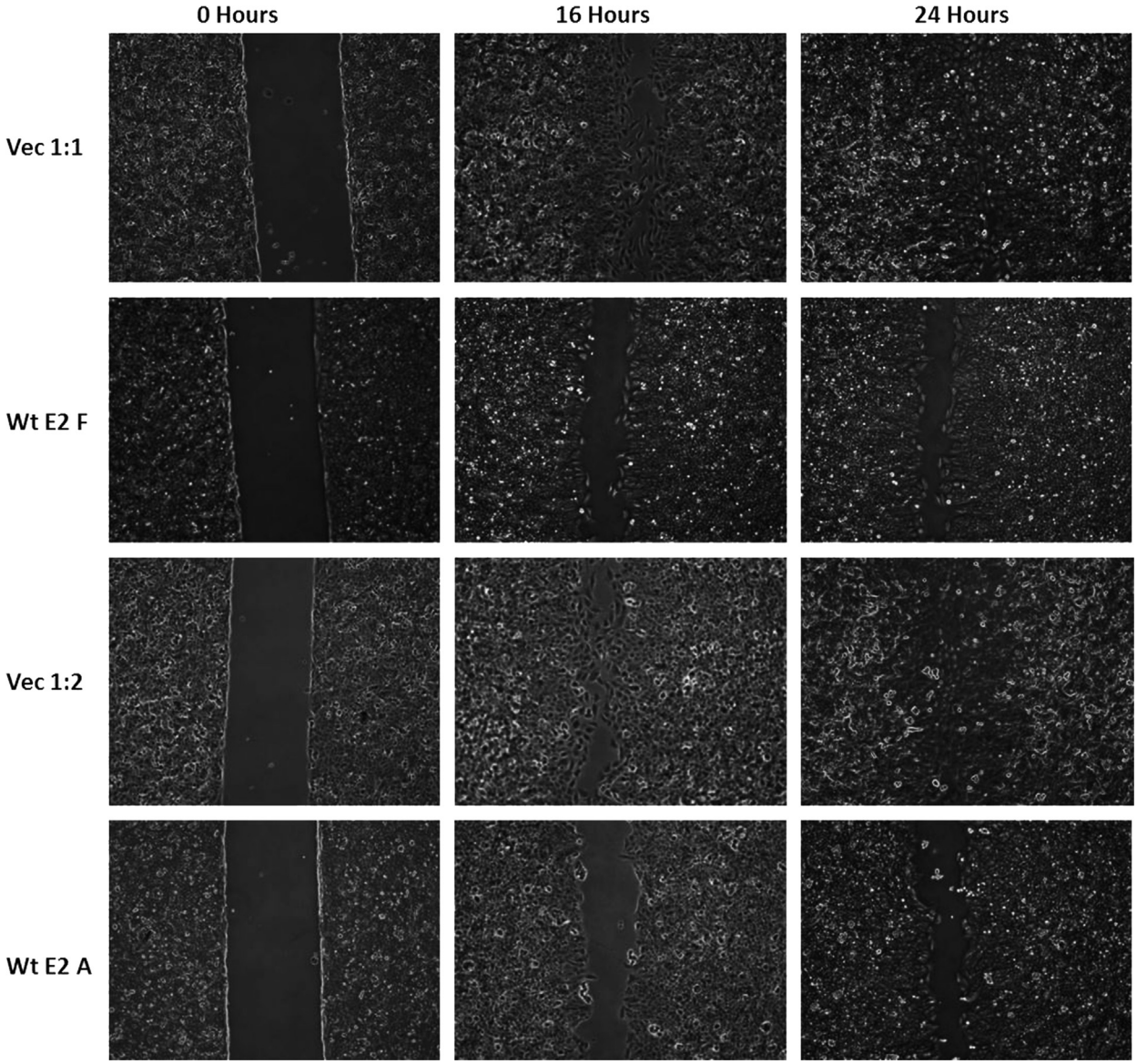

Fig. 3. HPV16 E2 expressing cells are delayed in their ability to "wound" heal. This is a representative panel of images from a wound healing experiment that was carried out three times with essentially the same result. For both of the Vec clones the wound is substantially healed after 16 h and almost fully closed at 24 h. For both of the E2 clones significant gaps remain at 16 h and also at 24 h. The results demonstrate that the E2 expressing cells are delayed in their ability to close the wound. This is a result of alterations in cell movement as the E2 and Vec clones grow at a similar rate (Fig. 1b).

systems where over expression results in repression of E6 and E7 expression and reactivation of the p53 and pRb tumor suppressor pathways (Desaintes et al., 1997; Goodwin et al., 1998; Hwang et al., 1996; Johung et al., 2007; Morrison et al., 2011; Naeger et al., 1999; Parish et al., 2006; Thierry et al., 2004; Wu et al., 2000). Such reactivation results in cell cycle arrest and apoptosis and expression of E2 in these cells results in activation of genes and pathways involved in these processes. To our knowledge this is the first report detailing analysis of host gene regulation by physiologically tolerated levels of E2 proteins; therefore the target genes identified are not related to E2 induced apoptosis or growth arrest. This is also the first report, to our knowledge, demonstrating that E2 can regulate the splicing of several hundred cellular transcripts of host genes. Several of the genes regulated by E2 are involved in cellular movement, and the differentially spliced gene population also had a significant representation of genes involved in cell movement and motility. The E2 expressing cells grew similarly to the vector control cells (Fig. 1b) but had diminished cellular movement in wound healing assays (Fig. 3). We also investigated the expression of the E2 regulated genes during cervical cancer progression using micro-array data from previous reports with clinical samples and demonstrated that 20 of the 74 E2 regulated genes were regulated in a similar manner in at least one of three clinical data sets. Overall the results demonstrate that E2 can regulate the host genome at multiple levels by controlling cellular gene expression as well as splicing of cellular transcripts.

The mechanism that E2 uses to regulate transcription from the host genome is unclear. There are several thousand E2 binding sites present in the human genome, many of them adjacent to actively transcribed genes, but none of these genes are regulated in the presence of E2 even although the E2 protein associates with the predicted target sequences (Vosa et al., 2012). This suggests that E2 regulates the host genome not by binding to E2 DNA binding sites adjacent to the regulated gene promoters but rather via interaction with cellular proteins associated with the regulated promoters. Analysis of these promoters revealed statistically over expressed sequences putatively bound by cellular transcription factors, OBOX1 and PITX3. Both of these proteins are homeobox containing

Table 4
Regulation of E2 target genes in micro-array data from clinical samples. Three previous studies looking at gene regulation by HPV infected cells (Ng et al., 2007; Scotto et al., 2008; Zhai et al., 2007) were studies for alterations in genes regulated by E2 listed in Table 1. Column 2 details the fold changes listed in Table 1. Columns 3 and 4 list genes altered from one study investigating gene changes from low grade cervical squamous intraepithelial lesions (column 3) and high grade cervical squamous intraepithelial lesions (column 4), both compared with normal cervix (set 1). Columns 5 and 6 list genes altered from another study investigating gene changes from high grade cervical squamous intraepithelial lesions (column 5) and cervical squamous cell carcinomas (column 6) versus normal cervix (set 2). Column 7 summarizes results from interrogating a data set comparing gene expression from cervical squamous cell carcinomas versus normal cervix (set 3). Raw data are available at Gene expression omnibus (GEO) and the accession numbers are listed above each column. The fold changes and *p* values demonstrating the significance of the up and down regulation are presented in Table S6.

Gene	Fold regulation by E2	Low grade versus normal (GSE27678)	High grade versus normal (GSE27678)	High grade versus normal (GSE7803)	Carcinoma versus normal (GSE7803)	Carcinoma versus normal (GSE9750)
SLC14A1	−5.9					Down
CD33	−4.1					Down
CSTA	−3.5		Down	Down		Down
HLA-DPA1	−2.4	Down				
GCNT2	−2.3			Down	Down	
RPL23	−2.2					Down
AURKC	−2.1					Down
ADRB2	−2.1		Down		Down	Down
GOLGA8B	2.1		Up	Up	Up	Up
ZNF271	2.2	Up	Up			
CCDC99	2.2		Up		Up	Up
ARHGAP11A	2.2	Up	Up			
ARHGFIB	2.4			Up		
FAP	2.5		Up			Up
SERPINA3	2.7	Up	Up	Up		Up
APBA2	3.0		Up	Up		Up
FAR2	3.7					Up
ENG	4.9			Up		
TNFAIP6	4.9					Up
HOXB2	10.6	Up	Up	Up		

transcriptional regulators, Obox1 is ordinarily expressed in germ cells (Rajkovic et al., 2002) while PITX3 regulates neuronal development (Smidt et al., 2004). Homeo-box genes are de-regulated in cancer and E2 could regulate gene expression via these factors by binding to promoters through OBOX1/PITX3 and altering transcriptional control of these genes. We hypothesize that E2 can regulate the host genome via interaction with transcriptional co-factors that are targeted directly to host promoters by cellular DNA binding transcription factors. E2 is known to bind a host of cellular proteins and therefore there are many candidates for mediating E2 regulation of host transcription. For example, E2 can bind the histone modifying proteins CBP (Lee et al., 2000) and p300 (Kruppel et al., 2008; Quinlan et al., 2013) and these can regulate E2 function. E2 can also bind the chromatin associated factor Brd4; this protein is essential for E2 regulation of transcription from HPV and synthetic promoters that have E2 DNA binding sites so may also be involved in regulation of host genes via interaction with Brd4 located at host promoters (Schweiger et al., 2006). E2 also binds the cellular chromatin binder TopBP1 that is essential for HPV DNA replication control (Donaldson et al., 2012); TopBP1 can also regulate transcription of host genes therefore is an additional candidate for mediating E2 transcriptional regulation of the host (Liu et al., 2011; McPhillips et al., 2004). There are many other candidates that could be involved in the control of host gene expression by E2 and further work is required to identify which factors are involved.

The mechanism that E2 uses to regulate host gene splicing is also unclear. Previous reports have suggested that E2 may regulate the expression of splicing factors in U2OS cells (McPhillips et al., 2004); we do not observe this at the transcriptional level (Table 1). It seems more likely that E2 can interact directly with splicing factors in order to alter splicing of cellular genes and is consistent with known interactions between E2 and splicing factors (Bodaghi et al., 2009). It is entirely possible that the interaction between E2 and splicing factors regulates their stability. In addition, regulation of viral gene splicing by E2 has been observed (Johansson et al., 2012). Future studies will focus on understanding how E2 is altering the expression of exons within genes, this is the first report demonstrating such a regulation of the host genome by E2.

Regulation of the host genome by E2 could clearly facilitate the viral life cycle in several ways. Table 4 demonstrates that E2 can regulate genes altered in the progression of cervical lesions. This is the first report demonstrating alteration of host gene splicing by E2 therefore there are no comparisons that can be made. It is of note that the alteration in the expression and splicing of genes involved in cell movement and motility were detected and that the E2 expressing cells had a reduced cellular motility when compared with wild type cells. A recent paper demonstrated that E2 can up regulate cell motility but this was done in the background of HPV positive cells making it difficult to compare with the results presented here (Morrison et al., 2011). In cervical cancers the HPV genome is integrated in the majority of cases, resulting in loss of the E2 gene (Mair et al., 2014; zur Hausen, 2009). It has been proposed that this loss of E2 results in elevation of E6 and E7 expression as their promoter is no longer repressed; such elevation could contribute to genomic instability and the transformed phenotype. The control of cell motility by E2 could also be lost in these transformed cells when E2 expression is lost further contributing to progression to carcinogenesis. This loss of E2 control could be mediated by the absence of regulation of gene splicing, or due to altered gene regulation. It is possible that a sub-set of the changes in host gene expression by E2 can be inherited into daughter cells via epigenetic regulation following E2 loss, but also that there is another sub-set of E2 regulated genes whose regulation is lost upon the loss of E2 expression.

Future studies will focus on enhancing our understanding of how E2 regulates the host genome. This regulation is likely essential to manipulate the host genome to allow progression of the viral infection. We hypothesize that chromatin modifications at the E2 regulated promoters are responsible for alterations in gene expression. Understanding the precise modifications involved would present opportunities to reverse the control of the cellular genome by E2. Our understanding of the chromatin

modifications responsible for regulating gene expression increases exponentially; great efforts are being directed at being able to manipulate these modifications to alleviate and treat disease (Mair et al., 2014). Such an approach could be used to reverse the regulation of the host genome by E2 and assist with the disruption of the HPV life cycle. It is also possible that the modifications by E2 could be inherited epigenetically into daughter cells so even when E2 expression is lost, reversing the effect of E2 regulation of the host genome may still affect the HPV transformed cell. We propose the U2OS system as a tractable model to understand how E2 regulates the host genome at both a transcriptional level and at a gene splicing level.

Materials and methods

Generation of stable U2OS clones

U2OS cells were grown in Dulbecco's modified eagle medium (DMEM) with 10% Fetal Calf Serum and 1% (v/v) penicillin/streptomycin mixture (Invitrogen) at 37 °C in a 5% CO2/95% air atmosphere. Cells were passaged every 3–4 days. To make stable cell lines expressing E2 4×10^5 U2OS cells were plated onto 100 mm^2 plates. The following day cells were transfected using the calcium phosphate method, with 1 μg of E2 encoding plasmid DNA containing a G418 resistance gene (Bouvard et al., 1994). 48 h post-transfection, cells were treated with 0.5% trypsin EDTA (Invitrogen), and re-plated at several dilutions (1:5, 1:20 and 1:50) and fed with DMEM media containing G418 (Geneticin) at a concentration of 0.75 mg/ml. Cells were monitored and re-fed every 3–4 days with fresh G418 media, for 14 days post the initial G418 treatment. After this time isolated colonies of surviving cells were seen. These colonies were transferred using cloning rings to 6 well plates, and cultures were maintained in G418 medium. Candidate clones were then expanded into 75 cm^2 flasks. The presence of E2 was tested using western blotting.

Western blotting

Cells were trypsinized and then washed twice with phosphate buffered saline. Pellets were then re-suspended in 100 μl lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150 mM NaCl, protease inhibitor cocktail (Roche)). The cells were then lysed on ice for 30 min, and the lysates cleared (10 min at 20,800 rcf at 4 °C). The supernatant was then removed to a clean tube. Protein levels were determined using a BCA Assay (Sigma).

30 μg of cell lysate for each sample was prepared and loaded onto a 4–12% gradient gel (Invitrogen), electrophoresed at 200 V for 1 h and transferred onto nitrocellulose membrane. Membranes were incubated in blocking buffer (5% NFMP, 0.1% Tween-20, PBS) at RT for 1 h then incubated with TVG261 mouse anti-HPV16 E2 antibody diluted 1:10 in blocking buffer. Membranes were washed in PBS-0.1%Tween then incubated with secondary antibody (anti-Mouse Ig, Horseradish Peroxidase linked) (GE Healthcare) diluted 1:50,000 in blocking buffer at RT for 90–120 min. Following another wash with PBS-0.1%Tween the chemiluminescence was developed using ECL-Plus (Amersham Biosciences) and the membrane exposed to film.

Growth curve

2×10^5 cells were seeded in 100 mm^2 plates in triplicate and grown in complete DMEM medium; cells were trypsinized and counted using a hemocytometer. This process was repeated three times in 3 days intervals. Growth curves of two separate sets of U20S vector and 16E2 wild type clones were plotted on a log scale from the successive cell counts, 0, 3, 6 and 9 days.

RNA preparation

1×10^6 stably transfected U20S cells were plated out on 100 mm plates. The following day cells were washed 2× with PBS. 600 μl of buffer RLT from the Qiagen RNeasy kit was added to each plate and incubated at room temperature for about 5 min; cells were then scraped from the plate and buffer RLT/cell mix was added to a Qiashredder column (Qiagen) and centrifuged following the manufacturer's instructions to homogenize the sample. The Qiagen RNeasy protocol was then followed to extract RNA from the U2OS cells. DNA was removed using DNase treatment (Qiagen) on column.

Exon array

Three independent polyA+ RNA preparations were made from the same clone (U2OS E2 clone F) and converted to cDNA and analyzed using Affymetrix Gene Chip Human Exon 1.0ST Array. 1.5 μg of RNA was required for the analysis. The quantity and quality of the RNA were analyzed using the Agilent 2100 Bioanalyser System. cDNA preparation, the microarray assay, and primary analysis were performed in Glasgow Polyomics at the University of Glasgow using standard Affymetrix protocols. Briefly, the sense strand cDNA was generated from total RNA using the Ambion WT Expression Kit (Applied Biosystems) followed by fragmentation and terminal labelling using the Affymetrix GeneChip WT Terminal Labelling Kit. The samples were then hybridized to the arrays using Hybridization Oven 640. The arrays were washed and stained using Fluidic Station 400 and scanned on the Gene Array Scanner 7G. The raw data in form of Affymetrix CEL files were generated from scanned images with GCOS software (Affymetrix). The raw data representing a selection of probes corresponding to the core annotation level were then normalized using GC-content by the Robust Multichip Average method followed by differential expression analysis using ANOVA module within Genomics Suite (Partek Inc.).

SYBR green qPCR

Sybr green real-time qPCR was used to validate the array gene expression results (DyNAmo SYBR Green qPCR Kit with ROX) using primers designed by Qiagen (Qiagen QuantiTech primer assay). The house keeping gene GAPDH was used as an endogenous control alongside the Vector, no E2 expressing U20S cell line to normalize the results using the ΔΔct method. A list of genes validated can be found in Table S1.

Identification of E2-regulated genes with over-represented DNA sequences within promoters

The promoter sequences within 1 kbp immediately upstream of the transcription start site of genes identified as regulated by E2 were analyzed for representation of all 7mer sequences. The hypergeometric distribution was used to assess *p*-values. Over-represented sequences were identified based on *p*-value and false discovery rate. Identification of potential transcription factor binding sites based on these sequences was performed using TOMTOM (Gupta et al., 2007).

Identification of genes with differential splicing in the presence of E2

The ratio of mean replicate expression data for the E2-expressing cells to the mean replicate expression data for vector control cells was calculated for each exon. The Log_2 transformed exon ratios were fitted to a normal distribution and statistically significant outlier exons were identified based on *p*-values and

false discovery rates. Genes with these exons represent proposed alternatively spliced transcripts. Exons expressing values near background in both E2-expressing cells and vector control cells were excluded from the analysis.

Identification of genes differentially expressed during cervical cancer progression

Expression of E2-regulated genes was analyzed in the following datasets of cervical SCC samples with published gene expression profiles: 32 samples of lesional epithelium microdissected from cervical high-grade squamous intraepithelial lesions (HSILs) and low-grade SILs (LSILs) and 12 samples of normal ectocervical squamous epithelium (set-1) (Ng et al., 2007); 21 cervical SCC, 7 HSIL plus 10 normal cervix (set-2) (Zhai et al., 2007); and 32 cervical SCC plus 21 normal cervix (set-3) (Scotto et al., 2008). All microarray data were obtained from the GEO database, with the accession numbers GSE27678, GSE7803 and GSE9750. We used ANOVA, with a post-hoc analysis by the Student–Newman–Keuls' test to determine genes differentially expressed during cervical cancer progression (fold change $>$ 1.5-fold and p value $<$ 0.05).

Wound assays

To determine cell migration, "wound-healing" (scratch) assays were used. Cell culture inserts (Ibidi, cat # 80209) were attached to 6 well plates and seeded with 5×10^4 cells in each side of the cell insert divide. This was done in replicate for each Vector and 16E2 wildtype U2OS clone. Cells were grown for 24 h until each side of the chamber was confluent. The cell insert was removed and images captured at time points 0, 16 and 24 h (Zeiss, Axiovert 200 M microscope and Axiocam). The width of the cell free gap was approximately 500 μm ($\pm$ 50 μm) at time 0 h; measurements were performed using Axiovision software at multiple points of the wound for each time point. These measurements were used to calculate the average distance the cells moved over a 24 h period. An average of three separate experiments was taken.

Wound assays – live cell imaging

8-well chamber slides were seeded with 5×10^4 cells in each well. This was done in replicate for each Vector and 16E2 wildtype U2OS clone. Cells were grown for 24 h until confluent. A scratch was made in the center of each well, using a 20 μl pipette tip. Cells were imaged over time, using a Zeiss AxioObserver Z1 microscope equipped with an Axiocam MRm CCD camera, a motorized XY stage that permits multi-point imaging and a Pecon stage incubation system to maintain cell viability. For these scratch assays, a $10 \times$ /0.3 EC Plan-Neofluar Ph1 objective lens was used. Zeiss Zen Blue software was used to program the system to record images from each well at 5-min intervals for 48 h. The halogen lamp was shuttered between time points and a green filter was placed in the optical path to minimize the effects of photo-toxicity. By necessity the results from these experiments are supplementary figures.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.022.

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